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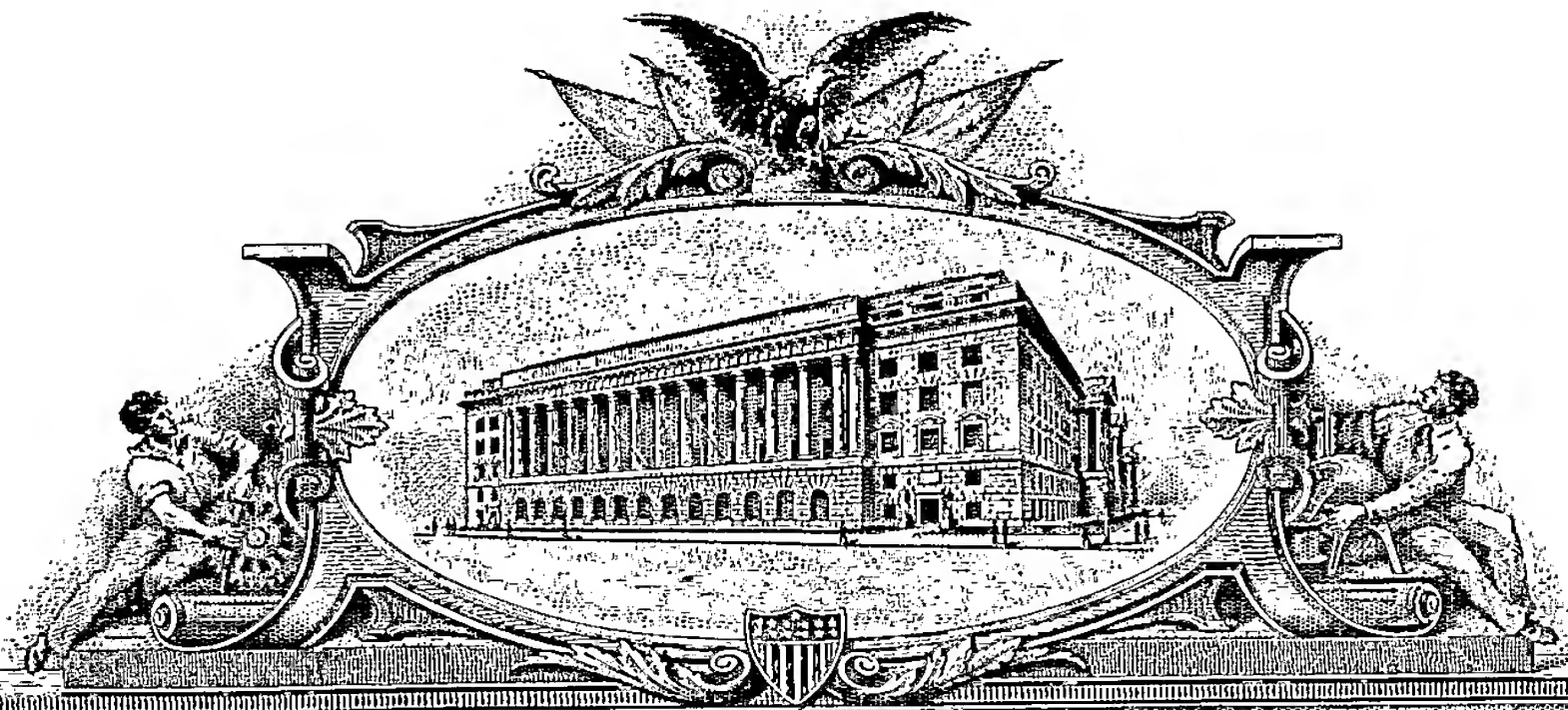Document type: Certified copy of priority document

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PROVISIONAL APPLICATION FOR PATENT COVER SHEET
This is a request for filing a PROVISIONAL APPLICATION FOR PATENT under 37 CFR 1.53(c).

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TITLE OF THE INVENTION (280 characters max)
A METHOD FOR DESIGNING SURFACES

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Respectfully submitted   Date **December 12, 2003**
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A METHOD FOR DESIGNING SURFACES

The present invention relates to a method of designing a substrate surface which has desirable properties in terms of its ability to bind or capture target molecules of interest.

5 More specifically, the present invention relates to a computer implemented method for molecular modelling of surface coatings, the characteristics of which are designed to bind molecules in some preferred orientation. The invention also relates to a method of producing such surfaces involving the method of design, and to surfaces when so-produced.

10 **Background**

In the life sciences, isolation of specific biomolecules of interest from complex mixtures and assays to identify those molecules and their interacting partners are commonplace. Such methods tend to be performed on solid phase substrates, normally made of glass,

15 silica, or plastics, such as polypropylene and polystyrene, and to increase throughput and improve efficiency, these substrates are typically used in the form of small beads, columns, microscope slides, multi-well plates or membranes. The basic assumption has always been that the surface characteristics of the substrate does not seriously affect the various interactions that are required to take place during the screening or separation process.

20 However, this is not necessarily the case and the lack of suitable solid phase substrates has lead to non-optimal processes and, in some cases, failure of the processes to work at all. For example, it is known that immobilization of proteins on plastic substrates such as latex beads and polystyrene multi-well plates can lead to conformational changes in the protein resulting in poorer than expected signal to noise ratio and sometimes complete failure in

25 the assay.

To generate a greater diversity of surfaces for new applications or to improve the performance of existing materials, different surface coatings have been applied to materials such as glass and plastics. A common strategy has been to identify surface coatings with

30 minimal non-specific binding and the potential to covalently bind target molecules that are subsequently used to capture their complementary binding molecules of interest. The target

molecule is usually a biological molecule, as synthetic alternatives do not normally have the required specificity and selectivity.

5   An important category of target molecules for separations and assays are antibodies and there are a number of methods to immobilize antibodies onto a substrate (see Ed Harlow and David Lane; Antibodies: A laboratory manual by Cold Spring Laboratory, (1988)). Covalent attachment of antibodies to a solid substrate surface can be categorized into three broad classes, as follows.

10   In the first class, protein A or protein G is first covalently attached to a substrate to act as a capture molecule for the antibody. The antibody requires this capture molecule to bind it to the substrate surface and this interaction is stabilized by cross-linking with a bifunctional coupling reagent such as dimethylpimelimidate (DMP). As both protein A and protein G bind to the Fc region of the antibody, the antigen binding site of the bound

15   antibody will be oriented correctly for optimal subsequent interaction with antigens. This technique tends to be expensive and initial coupling of protein A or protein G onto the solid support is random, leading to uncontrolled orientation and non-optimal antibody loading due to a limited number of protein A or G molecules being bound to the substrate in an orientation which is suitable for antibody binding.

20

A second type of coupling method uses substrate surface coatings having reactive groups that directly couple certain amino acid side chains in the antibody such as lysine. The main disadvantage of this approach is the lack of control on which lysine(s) in the antibody is/are coupled to the substrate surface. Poor orientation and damage to the antibody are

25   likely outcomes.

A third technique involves activating the antibody first and then coupling the antibody onto a substrate having some reactive groups on its surface. This technique has the same disadvantages as the previous method except when periodate is used to activate the

30   antibody. The periodate breaks the sugar rings in the Fc region and allows the antibodies to

be coupled to the substrate bound reactive groups such as amines.   In this case, orientation of the antibody can be controlled.

In the prior art, there are many examples on the use of small molecule ligands to bind or
5   capture proteins. For example, the strong binding affinity of biotin to streptavidin can be used.   However, if biotin is coupled onto the substrate surface than the antibody needs to be fused or coupled to the streptavidin sequence which greatly complicates the process.

As another example, glycogen synthase kinase-3 (GSK-3) inhibitors have been coupled
10   onto substrate surfaces to identify their actual intracellular targets (Knockaert *et al.*); (Identification following affinity purification on immobilised inhibitor; J. Biol. Chem. 2002 277:25493-25501). As another example, Schreiber *et al.* have synthesized a library of over 2 million unique chemical compounds on small latex beads to screen against cells and multiple proteins. The researchers also claimed printing such compounds on to glass
15   slides, creating small molecule microarrays to probe potential protein targets (Target-oriented and diversity-oriented organic synthesis in drug discover; Science 2000, 1964-1969). However, the experience of most laboratories is that the ligands identified from screening some kind of library invariably binds to native interaction regions of the target protein. If the objective is to orient such interaction regions of the target protein, then
20   existing approaches are very limiting.

As well, just because a protein is bound to the substrate surface through some small molecule ligand does not necessarily mean that the protein will remain in its preferred orientation and conformation. As mentioned before, non-optimal surfaces can lead to
25   conformational changes in the protein resulting in poorer than expected signal to noise ratio and sometimes complete failure in the assays.

Whether by covalent coupling or passive immobilization, there is a need to develop synthetic surface coatings that stabilize and maintain a biological molecule in some
30   preferred orientation. The use of another biological molecule (e.g. protein A) to orient the target molecule (e.g. antibody) only shifts the problem.

There are now highly parallel or combinatorial processes that can potentially generate millions of different but related surface coatings. For instance, published International patent application WO 03/095494 describes a way of assembling a large library of molecular coatings. More precisely, a thin reactive polymer layer is formed on top of a non-reactive substrate and, from that layer, it is possible to generate a wide diversity of molecular coatings by chemical transformation of one or more of monomer units. For instance, employing commercially available building blocks of amines and carboxylic acids, millions of surfaces can be generated from one starting point. When combined with further derivatization, where each generated surface can itself be propagated, extensive surface coating diversity is available. However, the ability to generate a vast number of different surface coatings does not in itself increase the success rate of identifying useful surfaces. Key solid phase applications not only require surfaces with high and low non-specific binding capabilities but also specific binding characteristics that may preferentially orient a target molecule such that some other part of the molecule is freely accessible for subsequent interaction with its complementary binding molecule. Another example in bio-separations is specific high binding capacity but efficient release under some slightly different conditions, e.g., pH or salt change. To efficiently identify such surface coatings, there must be some design elements to complement the capability to assemble and screen millions of surfaces.

Computational chemistry, which incorporates a variety of different methods developed and applied since the early 1980s, is now a well-established approach to identifying new drug leads in the pharmaceutical industry. The main focus has been on generating methodologies and computer programs to design potential small molecule compounds that would bind into a protein binding site or prevent a protein-protein interaction. Biological and chemical databases, virtual screening, pharmacophore modelling, 3-D molecular modelling, QSAR, structural prediction of homologous proteins, to cite a few, are routinely used techniques and have successfully led to the design of new drugs such as HIV protease inhibitors for treating AIDS (Leon *et al.*; Approaches to the design of effective HIV-1 protease inhibitors, Curr. Med. Chem., 2000, 7, 455).

At the early stage of drug design, computational chemists improve decision-making and help to accelerate the discovery process by increasing the speed and decreasing the cost of identifying lead compounds. This can be achieved by eliminating unpromising compounds

5 and/or by identifying ones which fulfill some criteria that have been identified as important for biological activity. This virtual screening can be performed mainly by using QSAR type models, pharmacophore models, and/or docking techniques. There are many variations on the theme and the choice of the techniques and all their combinations will mainly depend on the number of candidate molecules to be virtually screened and on the

10 knowledge of the target. In brief, the approach involves selecting compounds that fit a feature, a ligand or a receptor.

Assembling and identifying surfaces with the correct set of functionalities in their correct spatial distribution for any particular target protein is a time consuming process. To

15 address this challenge, the present invention seeks to provide computer-based methods for designing structural features on an artificial surface to capture and manipulate the orientation of different molecules, such as protein classes, and where the surface coating can preferentially enhance specific orientation of those molecules. With respect to the life sciences, these methods are intended to enable identification of optimised surfaces for new

20 bioassays as well as greatly improve the performance in existing bioassays.

Summary of present invention

In one embodiment, the present invention provides a method of designing a binding

25 surface for a target molecule having a functional binding site, which method comprises:

    (i)   identifying within the target molecule an anchor site which is remote from the functional binding site;

    (ii)   generating a pharmacophore model for the anchor site;

30   (iii)   using the pharmacophore model to identify an anchor site binding ligand; and

    (iv)   providing the anchor site binding ligand on a surface of a substrate such that the

ability of the anchor site binding ligand to bind to the anchor site is preserved.

In the context of the present invention the term "target molecule", and variations thereof such as "target protein", refers to a molecule which is bound to a substrate surface in some

5 preferred orientation so that the molecule has the ability to undergo a subsequent binding interaction with another molecule of interest. In the present specification the molecule of interest is termed a "complementary binding molecule". By way of illustration, in a biological assay, the target molecule may be an antibody and the complementary binding molecule an antigen.

10

As will be explained, the present invention uses molecular modelling techniques in order to design a substrate surface which has the potential to bind target molecules to maximise a predetermined orientation of those molecules. The ability to control the orientation of such molecules provides advantages in terms of sensitivity and resolving power when the

15 surface is used subsequently in order to utilise subsequent binding interactions of that molecule which are orientation dependent, such as to bind a complementary binding molecule of interest. As explained, conventional techniques for providing target molecules on a substrate are somewhat hit and miss in this regard. When compared to such techniques the surfaces designed in accordance with the present invention may have the

20 ability to bind a higher proportion of target molecules that more effectively bind to complementary binding molecules. This comes down to the ability to control the orientation of a target molecule on a substrate surface through surface design so that the target molecule is suitably orientated for subsequent binding interaction with its complementary binding molecule.

25

The invention also provides a method of producing a substrate including a target molecule bound to its surface in a predetermined orientation, the substrate having been designed in accordance with design method of the invention as described herein. The invention further provides substrates which have been designed in accordance with this method of design,

30 and their practical application.

Detailed discussion of the present invention

The present invention will be described with particular reference to designing polymeric surfaces that preferentially bind biological target molecules such as proteins, and typically

5   antibodies, in a predetermined orientation for use in immunoassays. However, it will be appreciated that the underlying concepts of the present invention may be applied to the design and manufacture of different types of surface which are required to immobilise other types of target molecule in some preferred orientation. This said, it is envisaged that the present invention will have primary applicability to the design of synthetic biomimetic

10   surface coatings.

For the purposes of the invention, the target molecule which it is intended to be immobilized to a substrate surface has two distinct types of binding site which are referred to herein as an anchor site and a functional binding site. The function and relative position

15   of these sites is fundamentally important in the present invention. The anchor site facilitates attachment of the target molecule to the substrate surface thereby enabling the target molecule to be immobilised for some specific assay. The functional binding site is responsible for the target molecule having some desired functionality by enabling the target molecule to undergo a binding interaction with its complementary binding molecule

20   while immobilised on the substrate. For the purpose of the invention, there can be two or more different or similar functional binding sites and it is also possible that an anchor site in one context may be a functional binding site in another context.

The interaction between the target molecule and its complementary binding molecule is

25   specific to the functional binding site and this means that when the target molecule is bound to the substrate, the functional binding site must be orientated in such a way as to be available for subsequent interaction with its complementary binding molecule. This has implications with respect to the relative position of the anchor site and functional binding site on the target molecule, and herein the term "remote" is intended to mean that the

30   spatial positioning of these sites within the target molecule is such that the ability of the functional binding site to interact as desired is preserved when the target molecule is

immobilised on a substrate via the anchor site. The term "remote" is not intended to mean that the anchor site and functional binding site are positioned on "opposing sides" of the target molecule, although this is obviously a possibility. The anchor site and functional binding site may occupy any position relative to each other provided the desired binding

5 potential of the functional binding site remains intact. In the context of an antibody as a target molecule, the Fv fragment corresponds to the functional binding site. The anchor site may be located on the Fc fragment of the antibody.

The first step of the method of the invention involves identifying within the target

10 molecule an anchor site in order to enable the target molecule to be attached to the surface of a substrate. The extent to which the target molecule is bound to the substrate surface must be sufficient such that the target molecule is not accidentally displaced during practical application of the surfaces designed and produced in accordance with the present invention. In principle, it is possible that the required degree of binding may be achieved

15 through a single anchor site. However, generally, the nature of the interaction which facilitates binding of the target molecule to the substrate through the anchor site is relatively weak and this means that binding one or a number of different ligands through one or a number of anchor sites is required to achieve suitable immobilisation of the target molecule. Thus, subject of course to context, references herein to a single ligand or a

20 single anchor site should be read as also meaning at least two such ligands or sites.

The location of suitable anchor sites is predicated by the location within the target molecule of the functional binding site, and this in itself will be known for the target molecule of interest. Indeed, the target molecule will be selected based on the nature of

25 this site and, more specifically, on the complementary binding molecule to which the functional binding site has binding specificity. It is possible based on the location of the functional binding site to determine possible anchor sites which will provide the functional binding site in a suitable orientation when the target molecule is immobilised on a substrate surface. In practice, potential anchor sites may be identified based on an

30 understanding of the molecular architecture of the target molecule and on the binding characteristics of the functional binding site, both of which may be well documented for a

given target molecule.

The experimental 3-D structure of the target molecule obtained by x-ray diffraction or NMR spectroscopy techniques is possibly the best source of information for this step of the
5   modelling. Both published and proprietary databases may be used in this regard. For instance, the Protein Data Bank (PDB) is the largest worldwide repository for the processing and distribution of 3-D structure data of large molecules such as proteins. In the absence of such experimental structure, homology modelling may generate a software-based 3-D model of the target molecule. For example, for a target protein this may be
10   done using its amino acid sequence and relating that to the structures of known proteins.

It may also be appropriate to undertake a bioinformatic search of relevant databases to search for the presence of potential anchoring sites. For example, public or proprietary databases of protein motifs or domain such as NCBI Dart, Smart, Pfam, Prosite, Interpro or
15   Blocks may provide data and tools to identify which domains are present within the target molecule (Marchler-Bauer *et al.*, CDD: a database of conserved domain alignments with links to domain three-dimensional structure. Nucleic Acids research **30** 281-283 (2002)). Analysis of protein-protein interaction screening data experimentally generated, for example, using yeast two-hybrid screens, may also provide information on which
20   anchoring sites are present within the target molecule.

Possible anchor sites may also be identified by computer modelling of the 3-D structure of a given target molecule. One skilled in the art would be familiar with sources of such information and with the kind of computer hardware/software that may be employed.
25   However, while ligand active sites can be identified, for example, by use of the Grid, MCSS, superstar, Q-fit programs or the Sphgen module from the Dock computer programs suite, identifying binding sites on a protein surface is recognized as being a difficult task. Indeed, it has been shown that a binding site present at the surface of a protein may be practically indistinguishable from other patches on the protein surface. Palma *et al.*,
30   (BiGGER: a new (soft) docking algorithm for predicting protein interactions; Proteins, 2000 Jun 1;39(4):372-84) describe the use of BiGGER, a soft docking algorithm for

predicting protein interactions based on the three-dimensional structures of unbound molecules. Recently, Ma *et al.*, (Protein-protein interactions: Structurally conserved residues distinguish between binding sites and exposed protein surfaces, PNAS 2003 100: 5772-5777) have demonstrated that the use of polar residue hot spots can be used to
5   determine potential binding regions.

Not all possible anchor sites identified in this step may ultimately be useful for binding the target molecule to the substrate surface and it is therefore usually necessary to identify a number of different anchor sites at various locations on the target molecule. This also
10   affords design flexibility.

Subsequent to identifying a suitably positioned anchor site on the target molecule, the method of the invention involves generating a pharmacophore model for that anchor site. In the context of the present invention the pharmacophore model is a set of spatially
15   distributed properties or centres that are likely to be responsible for the ability of a binding site (in this case the anchor site) to undergo some form of binding interaction. The pharmacophore model involves molecular features that relate to any form of interaction through which a binding site has binding potential, for example, hydrophobic, electrostatic and hydrogen-bonding interactions. The pharmacophore model characterises a particular
20   binding site by reference to such molecular features.

The pharmacophore model is a 3-D representation of molecular features and, as such, must be defined by reference to at least four centres (spatially distributed properties). It may aid flexibility of design to use pharmacophore models that are characterised by more than four
25   centres as this brings with it a greater number of candidate anchor site binding ligands which may interact with the anchor site as required.

The pharmacophore model can be generated by reference to the molecular features of the binding site itself and/or by reference to the molecular features of a set of one or more
30   ligands already known to bind to the anchor site of interest. One skilled in the art would be aware of sources of information concerning complementary ligands for a given anchor site

of a target molecule. For example, a number of online resources are available for protein-protein interactions. The Biomolecular Interaction Network Database (BIND) stores descriptions of interactions and molecular complexes such as between proteins, nucleic acids and small molecules. The Dictionary of Interfaces in Proteins (DIP) is another

5   resource on interacting protein surfaces.

Numerous techniques for generating a pharmacophore model are known in the art and the invention does not reside in the selection of any particular technique. By way of example mention may be made of the following methodology and/or software systems: Catalyst;

10   Ludi, DISCO; HipHop; GASP, Chem-X, Think and HypoGen. One skilled in the art would have no difficulty in using any of the known techniques in the context of the present invention.

Once a pharmacophore model has been generated for an anchor site the method of the

15   invention involves using the pharmacophore model to identify an anchor site binding ligand. The intention here is to identify a ligand which maps or fits the pharmacophore model to some extent and which therefore has potential to bind to the anchor site. Previously cited programs and others available in the art can be used to perform the virtual screening. An important aspect of the present invention is that the ligand does not have to

20   match precisely the full pharmacophore model to be considered as a "hit" if the model is defined by reference to a large number of centres. At the very least the ligand must match the pharmacophore model with respect to at least four centres thereof in order to have potential to bind to an anchor site characterised by the model. Thus, if the pharmacophore model has been defined by reference to a large number of centres, it will be appreciated

25   that the number of potentially useful ligands that may be identified against the model will be increased. It will also be appreciated that if the pharmacophore model is defined by reference to a large number of centres, it may be possible to rank the likelihood of ligands exhibiting the necessary binding interaction based on the number of centres which the ligand matches. A ligand which matches a pharmacophore model with respect to a large

30   number of centres is likely to be more suitable than a ligand which matches the model in a more limited way.

With respect to this step of the method of the present invention it may be useful to resort to compound databases which generally correspond to a corporate collection of physically available compounds or compounds available externally from chemical compound suppliers. In this latter case, two types of libraries can be used. The first type originates from molecules that can be bought on a one-at-the-time basis. Individual supplier catalogue of compounds can be used or compilations such as the MDL's ACD (Available Chemicals Directory) or CambridgeSoft's ChemACX might be a more comprehensive source. For example, the ACD is a structure-searchable database of commercially available chemical compounds, with pricing and supplier information for over a quarter of a million research-grade and bulk chemicals from over 600 suppliers worldwide. The second type of library is a screening library from screening compound collection suppliers where the full library or part of it can be acquired. Compilations of screening libraries are also available like the MDL Screening Compounds Directory or CambridgeSoft's ChemACX-SC. Another source of information might be a virtual library corresponding to compounds generated by computer software (CombiLibMaker, Legion) from a list of reagent and a given chemistry.

Molecular modelling software and techniques known in the art may also be used to translate a particular pharmacophore model into suitable ligand structures. Ludi is an example of a program that offers a *de novo* technique that has been recently extended to work with larger databases of flexible molecules. Techniques known in the art for performing this particular step are well suited to designing relatively small ligands (molecules) and they cannot readily be extended to the design of surface biomimetics. The main reason for this is the nature of the binding interactions involved in the binding event for a given binding site. For proteins, at least, the average contact area is 800Å^2 and molecules that could complement such a large surface area are generally rare. For example, the average contact surface area offer to a protein surface by a set of 7,595 commercial mono-carboxylic acids is about 130Å^2 with a standard deviation of 55Å^2. Furthermore, molecules in the high range of surface area generally have a large number (in excess of 15) rotatable bonds (excluding terminal groups) and it is either not possible or

not practical to use current pharmacophore methodologies for processing the vast array of possible configurations that this brings with it. Thus, the anchor site binding ligands generated in this step are relatively small and simple molecules.

5   In reality it is not guaranteed that an anchor site binding ligand identified in accordance with the present invention will bind as desired to an anchor site. For instance, part of the ligand may collide with residues of the anchor site or one or more structural features in the candidate ligand may be incompatible with one or more functional groups of the anchor site. The technique which is adopted generates candidate ligands and the method of the

10   invention preferably also includes a docking step to ensure binding efficacy of an anchor site/ligand pair. This also allows ligands to be ranked according to binding affinity for an anchor site.

Docking may be performed by various techniques known in the art such as Dock, FlexX,

15   Slide, Fred, Gold, Glide, AutoDock, LigandFit, ICM, QXP. In the present invention, the at least four centres of the pharmacophore model are used to position the candidate ligand onto the anchor site. Then an extensive conformational search may be used to generate all potential configurations that are acceptable in terms of steric constraints. Scoring of the resulting generated complexes can be performed using either physical-based, empirical or

20   knowledge-based scoring functions. Physical-based scoring functions are based on atomic force fields such as Amber or CHARMM. Empirical scoring functions such as Score or Chemscore are based on physico-chemical properties such as hydrogen-bond counts and use several energy terms that approximate for example hydrogen bonding, hydrophobic interactions and entropic changes to estimate the binding free energy. The coefficient used

25   in each term are derived from fitting to known experimental binding energies for a variety of different protein-ligand complexes. Knowledge-based scoring functions, such as PMF or Drugscore are based on a statistical analysis of protein-ligand complexes. An individual free energy term associated with an interatomic contact may be determined from its frequencies in the database. The total binding free energy is calculated by the sum of

30   individual free energies of interatomic contacts. The various types of scoring function can be used to perform an energy minimisation of the complex structure. The minimiser will

adjust the position, orientation and exact conformation of the ligand within the anchor site. The flexibility of the target molecule or its anchor site may also be taken into account. With the first type of scoring function, molecular dynamic simulations with explicit solvent can be carried out and free energy perturbation (FEP) or thermodynamic

5   integration (TI) methods generally give a good estimation of the binding free energies. It is to be noted that the optimised complex may no longer fit the pharmacophore centres that were initially used to position the ligand. The result is an anchor site binding ligand which is predicted to bind to the anchor site.

10   The interaction between the anchor site and the complementary anchor site binding ligand is relatively weak and this means that a number of such interactions are required to immobilise the target molecule on the surface of a substrate. Thus, in practice, it is usually necessary to identify a number of anchor sites and complementary anchor site binding ligands for a single target molecule. The number of anchor site/ligand binding pairs that

15   will be required will depend on the precise nature of the relevant interaction for a given pair and the sum of such interactions for all binding pairs involved. In practice whether one has identified an appropriate number and type of anchor site/ligand binding pairs for a given target molecule may be determined by assessing whether the capture molecule is suitably immobilised on a chosen substrate.

20

The next step of the method of the present invention involves providing the anchor site binding ligand on the surface of a substrate. The ligand must be immobilised on the surface so that the target molecule may itself be immobilised. Furthermore, when multiple ligands are involved (as is normally the case in practice), the ligands must be provided on

25   the substrate surface with a suitable spatial distribution such that the ligands are suitably positioned to facilitate binding to the respective anchor sites of the target molecule. Thus, the spatial distribution of the individual anchor sites on the target molecule is also an important consideration as this will dictate the relative position of the respective anchor site binding ligands required on the substrate surface. One way of doing this is by

30   including the anchor site binding ligands as suitably positioned pendant groups on a backbone molecule which is bound to the substrate surface. Here the backbone molecule

serves to (indirectly) attach the anchor site binding ligands to the substrate in an orientation which will enable subsequent binding of each ligand to its complementary anchor site. Again, molecular modelling techniques may be used to design suitable backbone molecules. It will then be necessary to consider which designed structures may be

5 constructed in practice by techniques known in the art. Of course, when provided on the backbone the pendant anchor site binding ligands must retain the ability to bind to the anchor site of interest. This can be verified by screening using techniques mentioned herein.

10 In the prior art, low affinity ligands identified through experimental means have been tethered together through flexible linkers to form higher affinity ligands (D.J. Maly, *et al.* Combinatorial target guided ligand assembly: Identification of potent subtype-selective c-Src inhibitors., Proc. Natl. Acad. Sci., 97, 2000, 2419-2424; S.B. Shuker, *et al.*, Discovering high-affinity ligands for proteins: SAR by NMR , Science, 274, 1996, 1531-

15 1534.) The focus of such work was to develop small molecule drug candidates and not polymeric coatings.

In an embodiment of the present invention the anchor binding site ligands may be designed to be incorporated within the repeat units of a polymer that forms the substrate surface. In

20 its simplest form, the polymer is a homopolymer. Assuming multiple anchor site binding ligands are involved, the polymeric repeat unit will have at least two points of diversity based on the nature of the anchor site binding ligands which are included. The characteristics of the repeat unit may be derived from the monomers from which the polymer is formed, although the polymer may be formed and then modified to include

25 pendant anchor site binding ligands which impart desirable non-covalent binding properties. In the latter case the polymer must of course include reactive functionalities to enable subsequent reaction to introduce the anchor site binding ligands.

The anchor site binding ligands that bind to the target molecule may be components of

30 different repeat units in the polymeric chain but it is also possible that the ligands are within one repeat unit of the polymeric chain.

In one preferred embodiment, the polymer may be a copolymer of first and second monomers as described in published International patent application WO 03/095494. Here, examples of the first monomer include styrene (optionally substituted), dimethyl

5   acrylamide, acrylonitrile, N,N-dimethyl (or diethyl) ethyl methacrylate, 2-methacryloyloxy-ethyl-dimethyl-3-sulfopropyl-ammounium hydroxide, and methoxy PEG methacrylate.

The second monomer usually includes some functional group that may undergo a number

10   of chemical transformations. Examples of the second monomer include hydroxyethyl methacrylate, maleic anhydride, N-hydroxysuccinimide methacrylate ester, methacrylic acid, diacetone acrylamide, glycidyl methacrylate, PEG methacrylate and fumarates.

The repeat unit may be derived from more than two different monomers to provide a

15   polymer having a greater number of points of diversity in terms of binding ability as well as a greater diversity of repeat unit templates on which the anchor site binding ligands are arranged. In the following, for convenience, reference is made to a copolymer of first and second monomers only but additional monomer(s) may be present in the repeat unit.

20   As required, the polymer may also be modified by incorporation of a spacer between the copolymeric portion and the anchor site binding ligand. The spacer may be used to facilitate attachment of the anchor binding site ligand and further increase spatial distribution between the different anchor binding site ligands. Thus, the spacer will include a chemical group that is reactive towards the copolymer and a separate chemical

25   group that is reactive towards the anchor site ligand in question. Thus, the spacer may be represented by the formula X-Q-Y where X and Y are chemical groups that are reactive towards the copolymer and anchor site ligands respectively.

Typically, X and Y may be the residue of an amino, hydroxyl, thiol, carboxylic acid,

30   anhydride, isocyanate, sulfonyl chloride, sulfonic anhydride, chloroformate, ketone, or aldehyde, provided that X and Y are not reactive with each other or Q. Q is typically a

linear or branched divalent organic group. Preferably Q is selected from C_1 to C_{20} alkylene, and C_2 to C_{20} alkenylene, wherein one or more carbon atoms may be substituted with a heteroatom selected from O, S or N.

5   In alternative embodiments, the spacer group may have a branched structure whereby multiple functional groups may be attached at the ends of the branches. The spacer group may be attached to the copolymer and then reacted with the anchor binding site ligand. Alternatively, the spacer group may be reacted with the anchor binding site ligand and then this assembly reacted with the copolymer. The spacer may be modified with more than 10   one anchor site binding ligand.

The substrate may be formed of any material conventionally used in the intended field of application. For example, the substrate may be glass, silica or plastic. Suitable plastics materials include: nitrocellulose; polyolefins such as polyethylene, polypropylene and 15   polymethylpentene; polystyrene or substituted polystyrenes; fluorinated polymers such as poly(tetrafluoroethylene) and polyvinylidene difluoride; polysulfones such as polysulfone and polyethersulfone; polyesters such as polyethylene terephthalate and polybutylene terephthalate; polyacrylates and polycarbonates; and vinyl polymers such as polyvinylchloride and polyacrylonitriles.

20

The substrate may take any form. In biological applications the substrate will usually be in the form of beads, membranes, multi-well plates, slides, capillary columns or any other format that is used for biological assays, affinity separations, diagnostics or other applications where biological molecules are immobilised on some insoluble maternal 25   (solid support).

Generally, the polymer coating may be applied to the substrate using any of the vast assortment of surface modifications methods known in the art (e.g. dip coating, plasma polymerization, vapor deposition, stamp printing, gamma irradiation, electron beam 30   exposure, thermal and photochemical radiation).

In one embodiment, the polymer coating is graft polymerized from the constituent monomers on the substrate using chemistry well-known in the art. A wide range of polymerization processes present in the art may be utilized. For example, controlled and/or living polymerization techniques of cationic, anionic, radical (such as NMP, ATRP,

5   RAFT, Iniferter), condensation, and metathesis (such as ROMP and ADMET) all may be used. Non-controlled methods of polymerization well known in the art may also be utilized with this invention.

When the polymer includes a functional group and optionally a spacer group, these may be

10   introduced after the copolymer has been graft polymerised onto the surface of the substrate.

Alternatively, the polymer may be applied to the substrate as a polymer solution, comprising macromers that will allow tethering by complementary chemistry to the surface

15   of the substrate or encourage entanglement of the polymer in solution with the substrate surface. In the case of a macromer solution, the reactive units of the macromer may either be present at the end groups, or spaced throughout the polymer in a random, block, or gradient fashion.

20   Preferably, the polymer coating is polymerised from constituent monomers to provide an alternating or block copolymer. The alternating, or substantially alternating character, of the copolymer is believed to provide an important spatial arrangement of the constituent monomers. Those skilled in the art will understand the degree of regularity necessary in order for a copolymer to be considered of alternating character. It is preferred that the

25   alternating copolymer has an alternating character defined by greater than 70 % of consecutive comonomer residue units being alternate between residues of the first monomer and the second monomer, more preferably greater than 90%. The block nature of the copolymer may also vary in an alternating fashion.

30   It may also be possible to apply the polymer as a simple coating on the substrate without any covalent binding to the substrate surface. Conventional techniques, such as dip

coating, may be used. Crosslinking of the polymer may· be required for fixing on the substrate thereby preventing the washing off during use. The polymer may be provided on the membrane in ready to use form or it may be functionalised further, for example by introduction of the additional functional group as described above.

5

Alternatively, or in addition, data processing methods well known in the art may be used to control the processes involved in the present invention, including e.g. applying or polymerizing the backbone coating on the substrate, control of chemical reactions involved in further generating the synthon and/or the reactions and interactions occurring in, within

10 or between a population or array of surface coatings on a substrate.

Aspects of the present invention are illustrated in the accompanying non-limiting figures in which:

15 Figure 1 is a schematic illustrating the structure of an immunoglobulin (IgG2a) and its interaction with antigen molecules;

Figure 2 is a computer generated representation in which crystal structures of human IgG Fc fragment are superposed and where only the interacting regions of the proteins A and G

20 are displayed;

Figure 3 is a schematic showing how pharmacophore modelling may be carried out in practice using 4 centre pharmacophore keys;

25 Figure 4A is a computer generated representation showing the binding of two anchor site binding ligands to the anchor sites of a protein molecule ;and

Figure 4B shows schematically the attachment of two anchor site binding ligands to a surface through a polymeric backbone derived from styrene and maleic anhydride.

30

Embodiments of the present invention are illustrated in the following non-limiting example.

Example: Design of surface coatings for capture and display of antibodies

5

The present invention provides a method of designing and assessing binding surface for a given target molecule and has been applied in this example to the design of surface coatings for capture and display of antibodies. The method involves a series of steps as follows:

10

(a) **Identification within target molecule of an anchor site which is remote from the functional binding site**

Antibodies or immunoglobulins are host proteins produced by B-lymphocytes and plasma

15   cells in response to the presence of a specific antigen (foreign molecule) and are capable of reacting with that antigen. The fine-specificity of antigen recognition by monoclonal antibodies coupled with the relative ease of producing them has resulted in widespread use of monoclonal antibodies in both research and medicine.

20   IgG antibodies are among one of the five major classes of immunoglobulins that also include IgA, IgD, IgE, and IgM antibodies. Each antibody class is distinguished by certain effector functions and structural features. In some species, the immunoglobulin classes are further differentiated according to subclasses, adding another layer of complexity to antibody structure. In humans, for example, IgG antibodies comprise four IgG subclasses,

25   that is IgG1, IgG2, IgG3, and IgG4. Each subclass corresponds to a different heavy chain isotype.

Antibodies exhibit two fundamental types of structural variation (see Figure 1 below). Subtle structural differences in their antigen combining sites, or variable regions, account

30   for their unique antigen binding specificities. That structural unit is composed of two fragments, namely the Fab and the Fv fragments. In the context of the present invention

the functional binding site corresponds to the Fv fragment that binds the antigen. Structural differences outside the antigen combining sites, in the so-called constant regions, correlate with different effector functions mediated by antibodies, such as complement activation or binding to one or more of the antibody Fc receptors expressed on monocytes and granulocytes. The variable and constant regions of antibodies arise from distinct structural domains, such as the C_H2 and C_H3 domains for the Fc fragment. If bound to a solid surface through the Fc fragment, both Fv fragments will be oriented correctly for maximal interaction with the antigens. The anchor site (which is remote from the functional binding site) corresponds to the Fc fragment, namely the C_H2 and C_H3 domains (see Figure 1).

It is well known by those skilled in the art that both protein A and protein G bind to the Fc fragment of antibodies. Protein A has different affinities for antibodies from different species, classes and sub-classes. Interestingly, protein G has a different spectrum of binding affinities from protein A.

Protein A has a high affinity for human, pig, rabbit and guinea pig antibodies; a moderate affinity for horse, cow and mouse antibodies; and a low or no affinity for sheep, goat, chicken, hamster and rat antibodies. Protein G has a high affinity for human, horse, cow, pig and rabbit antibodies; a moderate affinity for sheep, goat, hamster, guinea pig, rat and mouse antibodies; and a low affinity for chicken antibodies. When using monoclonal antibodies, protein A has a high affinity for human IgG_1, IgG_2, IgG_3, for mouse IgG_{2a} and IgG_{2b}; a moderate or low affinity for mouse IgG_1 and IgG_3, for rat IgG_{2c}; and no affinity for human IgG_1, for rat IgG_1, IgG_{2a} and IgG_{2b}. Protein G has a high affinity for human IgG_1, IgG_2, IgG_3 and IgG_4, for mouse IgG_1, IgG_{2a}, IgG_{2b} and IgG_3, for rat IgG_{2a}; a moderate or low affinity for rat IgG_1, IgG_{2b} and IgG_{2c}.

Protein A is a 42,000 dalton protein that is a cell-wall-associated protein of *S. Aureus*. Protein A has five consecutive highly homologous domains that all present an IgG binding activity and has also a region that anchor the protein in the cell wall. The crystal structure of the Fc fragment of human IgG and it's complex with fragment B of protein A was solved to 2.9 Å resolution (J. Deisenhofer, Crystallographic Refinement and Atomic

Models of a Human Fc Fragment and its Complex with Fragment B of Protein A from Staphylococcus aureus at 2.9 and 2.8 Å Resolution. Biochemistry, 20: 2361-2370, 1981). The crystal structure is available at the PDB under the code 1FC2 (http://www.rcsb.org/pdb/cgi/explore.cgi?pdbId=1FC2).

5

Protein G is a 30,000 to 35,000 dalton protein isolated from the cell wall of *beta-hemolytic Streptococci*. Protein G has three (or 2) highly homologous domains named C_1, C_2 and C_3 (or B_1 and B_2) that are located at the C-terminal end of the molecule whereas an albumin binding region is present at the N-terminal part. The crystal structure of the Fc fragment of

10   human IgG and it's complex with fragment C_2 of protein G was solved to 3.5 Å resolution (Sauer-Eriksson, A.E., Kleywegt, G.J., Uhl, M., Jones, T.A. 1995. Crystal structure of the C2 fragment of streptococcal protein G in complex with the Fc domain of human IgG. Structure 3:265-278.). The crystal structure is available at the PDB under the code 1FCC (http://www.rcsb.org/pdb/cgi/explore.cgi?pdbId=1FCC).

15

From these crystallographic studies, it has been shown that both protein A and protein G bind to the Fc fragment in slightly different binding modes. The protein G:Fc complex involves mainly charged and polar contacts and is mainly located on the C_H3 domain, whereas protein A and Fc are held together through non-specific hydrophobic interactions

20   and a few polar interactions and the complex is located at the hinge that connects the C_H2 and C_H3 domains. Several residues of the Fc fragment are involved in both the protein G:Fc and the protein A:Fc complex, as shown by the superposition of both crystal structures and where only interacting region of the protein A and G are displayed (see Figure 2).

25

Due to their interaction with the Fc fragment, to their different spectrum of IgG binding affinities, to their close but different binding modes and to the availability of crystal structures, both the protein G:Fc and protein A:Fc complexes were ideal to generate pharmacophore models for the targeted anchor sites.

30

(b) **Generation of a pharmacophore model for the anchor site**

The pharmacophore models used in this example consist of the hydrogen bond donor feature (D or HDON), the hydrogen bond acceptor feature (A or HACC), the positive charge feature (P or POS), the negative charge feature (N or NEG) and the aromatic

5   feature (R or AROM) arranged in three-dimensional space.   When identified, pharmacophoric features are assigned to corresponding centres and stored in the coordinate system of the structure. The A, D, P and N centres are placed on the corresponding atoms using their coordinates. For R centre, a dummy atom is placed at the centre of the aromatic ring.

10

In order to have the same coordinate system for both models derived from the protein G:Fc and protein A:Fc complexes, the crystal structure of the protein A:Fc complex was overlaid on the crystal structure of the protein G:Fc complex using VMD 1.8 software. The overlay was performed based on the Calpha atoms for the residue that form the

15   binding sites (250-254; 310-315; 380-382; 428-438).

For both models, the binding sites were defined by selecting residues with at least one atom within 5Å of the binding protein. An hydrogen bond was considered if the distance between the acceptor and donor heavy atoms was less than 4.5 Å. The resulting models are

20   given below.

Model from the protein G:Fc complex:

ATOM	1	HDON	PHM	G	1	19.077	8.971	-4.248
ATOM	2	HACC	PHM	G	1	14.113	4.201	1.536
ATOM	3	HACC	PHM	G	1	17.918	3.486	1.481
ATOM	4	HDON	PHM	G	1	19.523	4.191	0.055
ATOM	5	HDON	PHM	G	1	22.973	3.731	-12.398
ATOM	6	POS	PHM	G	1	22.973	3.731	-12.398
ATOM	7	HACC	PHM	G	1	17.675	-3.346	-14.103
ATOM	8	HDON	PHM	G	1	20.027	-2.937	-4.790
ATOM	9	POS	PHM	G	1	20.027	-2.937	-4.790
ATOM	10	HACC	PHM	G	1	18.730	-4.692	-8.723
ATOM	11	NEG	PHM	G	1	18.730	-4.692	-8.723
ATOM	12	HACC	PHM	G	1	18.943	-4.570	-6.637
ATOM	13	NEG	PHM	G	1	18.943	-4.570	-6.637
ATOM	14	HACC	PHM	G	1	17.605	4.042	-7.871

```
ATOM        15 HACC PHM G   1      16.447    3.281   -2.678
ATOM        16 HACC PHM G   1      15.488    4.973   -0.044
```

Model from the protein A:Fc complex:

```
ATOM         1 HACC PHM A   1      18.604  -19.135    1.282
ATOM         2 HDON PHM A   1      13.943   -6.457   -8.546
ATOM         3 HDON PHM A   1      18.539   -4.313   -6.079
ATOM         4 AROM PHM A   1      18.717   -8.843   -1.743
ATOM         5 HDON PHM A   1      13.824   -0.683    1.203
ATOM         6 HDON PHM A   1      22.083   -2.071    4.645
ATOM         7 AROM PHM A   1      19.961   -3.084    3.111
ATOM         8 AROM PHM A   1      18.000    0.350   -6.650
ATOM         9 HACC PHM A   1      14.103   -2.673   -8.529
ATOM        10 HACC PHM A   1      20.108   -3.926   -4.484
ATOM        11 HACC PHM A   1      18.231  -18.189    3.308
ATOM        12 HDON PHM A   1      18.231  -18.189    3.308
ATOM        13 NEG  PHM A   1      19.175  -18.147  -10.778
ATOM        14 HACC PHM A   1      18.748  -19.432   -8.668
ATOM        15 NEG  PHM A   1      18.748  -19.432   -8.668
ATOM        16 HACC PHM A   1      18.559  -16.971   -8.656
ATOM        17 NEG  PHM A   1      18.559  -16.971   -8.656
ATOM        18 HACC PHM A   1      19.175  -18.147  -10.778
ATOM        19 HACC PHM A   1      15.931   -1.237   -9.617
ATOM        20 HDON PHM A   1      18.892   -3.019  -10.350
ATOM        21 HACC PHM A   1      18.539   -4.313   -6.07
ATOM        22 POS  PHM A   1      20.264  -13.684    6.915
ATOM        23 HDON PHM A   1      20.264  -13.684    6.915
ATOM        24 POS  PHM A   1      18.436  -15.464   -6.156
ATOM        25 HDON PHM A   1      18.436  -15.464   -6.156
```

The coordinates of the two models define the relative relationship between the centres and any rotation or translation of the coordinates cannot be interpreted as a different model. Also, proteins are flexible entities and the x-ray determination is not without errors. A tolerance of 2 Å for each centre can be allowed.

(c) Use of the pharmacophore model to identify an anchor site binding ligand

A) Anchor site binding ligand screening database

9,289 available mono carboxylic acids were extracted from the ChemFinder ChemACX2000 database and concatenated into an SD file. The CsNum was used as a

unique identifier. The SD file was converted into a SMILES file and aromatisation was applied (use of lower case aromatic notation in the SMILES string instead of a Kékule format). Any molecules with a salt, within mixtures, or with atoms other than F, O, N, H, C, Cl, S, Br, P, and I were removed, leading to a dataset of 7,595 compounds.

5

Since the compounds were extracted from supplier databases, it was assumed that the right stereochemistry at chiral centres and cis/trans double bonds was correctly depicted. Otherwise, the generation of all possible stereoisomers could have been performed. Also, for this example, no attempt was made to take into account the possible tautomeric or ionic forms of a given compound. There is no way to know *a priori* which tautomer is most likely to bind to the receptor, as the pH at the interface is unknown. It would have been preferable to include all the tautomers as possible structures. Stergen or Tautomer software could be used for that purpose.

In order to avoid excessive computational time in generating the conformers, any molecules with more than 10 rotatable bonds were removed, leading to a final dataset of 6,571 compounds.

B) Pharmacophore screening database

Due to the computational time required to determine pharmacophore features and to generate all plausible conformations within a molecule, the pharmacophore screening databases are generated once so that they can be re-screened later in other pharmacophore models.

The approach used here is inspired from the Think methodology. It uses the notion of 4 centre distance keys (see Figure 3 below). The first 4 letters of the key represent the nature (A, D, N, P or R) of the 4 centres that make the key. The order of the letters is determined by their alphabetical order. When a key is made from centres of the same feature, then the order is determined by a set of rules based on their relative distances to other centres or between themselves. The next 6 digits of the key encode the 6 distances between the 4

centres. The distances d1, d2, d3, d4, d5 and d6 are always defined in the same way. "d1" is the distance between the first and the second centres. "d2" is the distance between the first and the third centres. "d3" is the distance between the first and the fourth centres. "d4" is the distance between the second and the third centres. "d5" is the distance between

5   the second and the fourth centres. "d6" is the distance between the third and the fourth centres. A letter code (0, 1, ..., 9, a, ...z) is associated to each distance using a binning scheme. For example, a bin "0" means that the distance between the two centres is less than 3 Å; a bin "4" means that the distance between the two centres is between than 6 and 7 Å. The binning scheme can be changed but this implies rebuilding the pharmacophore

10   screening database.

One key encodes a set of four centres for a given conformation. For a given molecule, all possible combinations of 4 centres need to be generated and all possible conformation need to be considered. The pharmacophore definition of a molecule can be viewed as the logical

15   "OR" of all the keys thus generated.

For conformational sampling, a systematic search was performed using an increment of 120 degrees for a sp^3-sp^3 bond, 60 degrees for sp^3-sp^2 bonds and 180 degrees for sp^2-sp^2 bonds.

20

A pharmacophore screening database for the 6,571 mono-carboxylic acids was generated using a binning scheme of 11 bins with the following limit: <3, <4, <5, <6, <7, <9, <11, <14, <17, <20 and >20. The pharmacophore perception failed for some molecules leading to a final of database of 5690 compounds described by 8,685,484 pharmacophore

25   "screening" keys.

C) Screening of candidate anchor binding site ligands against the pharmacophore models

Using the same methodology as described for generating the pharmacophore screening

30   keys, pharmacophore "query" keys were generated from the pharmacophore models derived from the protein G:Fc and protein A:Fc complexes. All possible combinations of 4

centres are generated but only one conformation corresponding to the model is used. 1,462 and 12,650 pharmacophore query keys were generated from the 16 and 25 centres of the protein G:Fc and protein A:Fc models respectively.

5   For each candidate molecule of the screening database, a score of 1 is added to the molecule score each time one of its pharmacophore screening key matches a query key. Molecules with at least a score of one can be considered as a hit, but the higher the score, the better the molecule may complement the anchor site. In the present design, 1,133 and 1,051 compounds gave a score greater than one, but only compounds with a score greater

10   that eleven were further considered, leading to the selection of 173 and 168 compounds based on the protein G:Fc and protein A:Fc models respectively.

Compounds selected from the pharmacophore screening were docked onto the rigid anchor site based on each matched key. For each configuration, a conformational analysis is

15   performed to remove conformations where atoms from the ligand collide with atoms from the anchor site. Plausible complexes were scored using the ChemScore function. (M.D. Eldridge, C.W. Murray, T.R. Auton, G.V. Paolinine, and R.P. Mee J. Computer-Aided Molecular Design 11:425-445 (1997).)

20   A visual inspection of the complexes was performed to check the availability of the carboxylic acid used for coupling to the linker while keeping the binding characteristics. For the retained configuration, the difference in AM1 intramolecular energies between the bound and the optimised unbound ligand was taken into account. Configurations where the difference was greater than 15 kcal/mol were rejected.

25

Table 1 below gives some examples of 20 compounds of interest selected from the protein G:Fc complex hit list. Figure 4A below display two of these ligands bound to the C_H3 domain and Figure 4B is a schematic representing a monomeric unit of the surface coating with these two ligands.
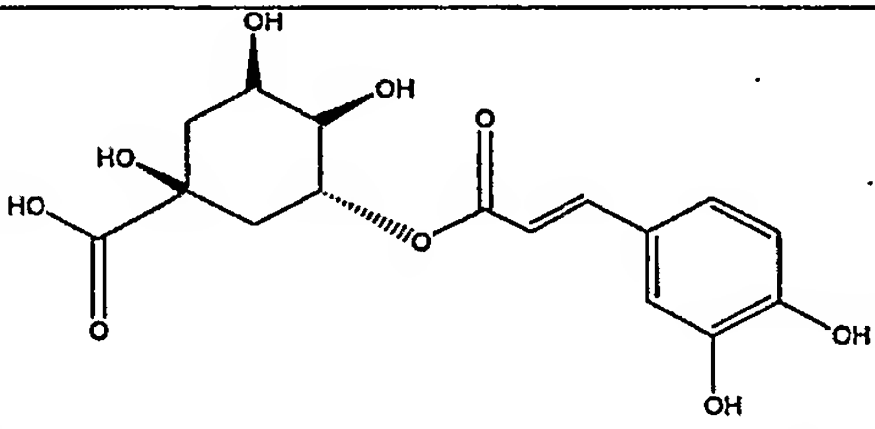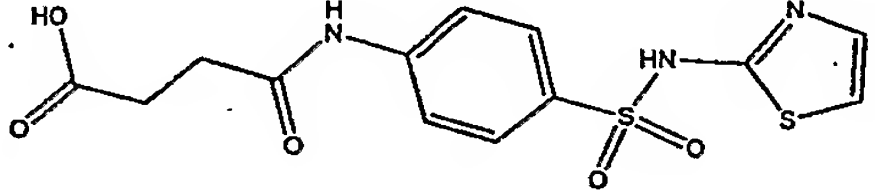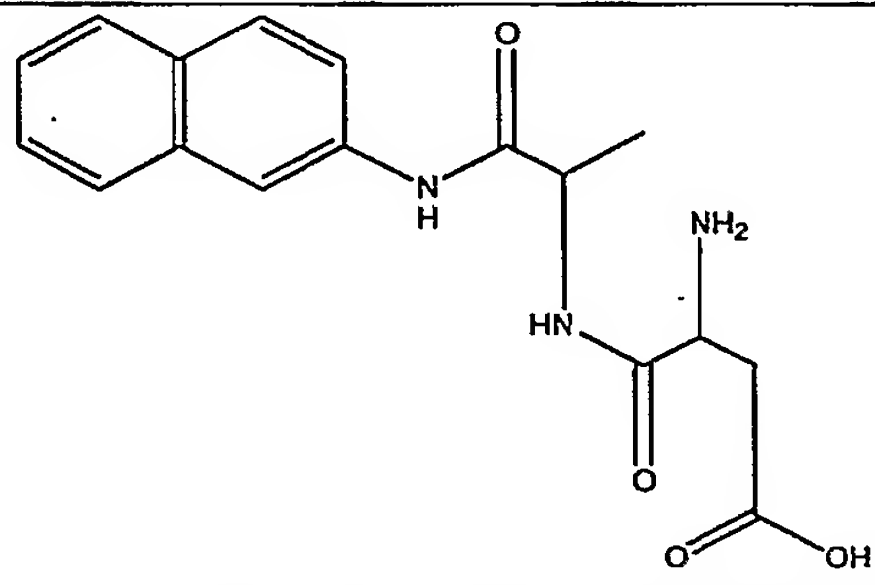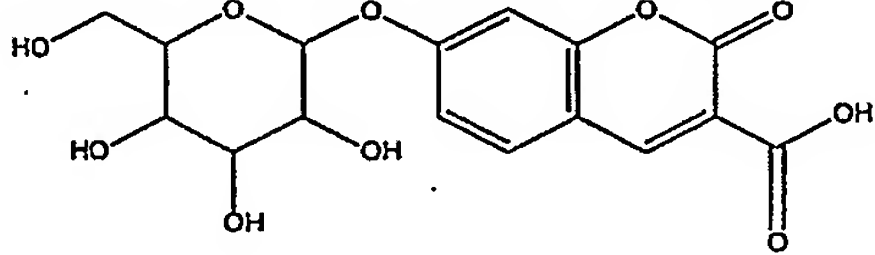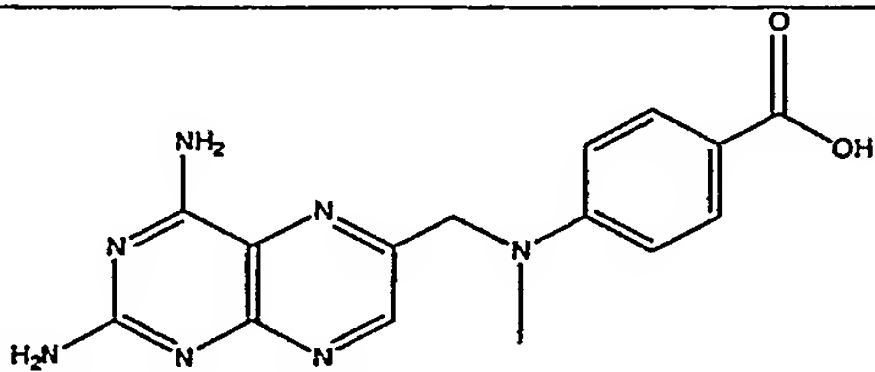
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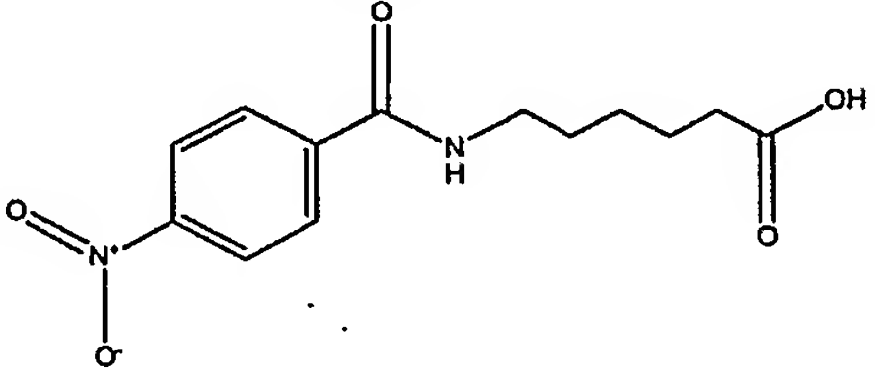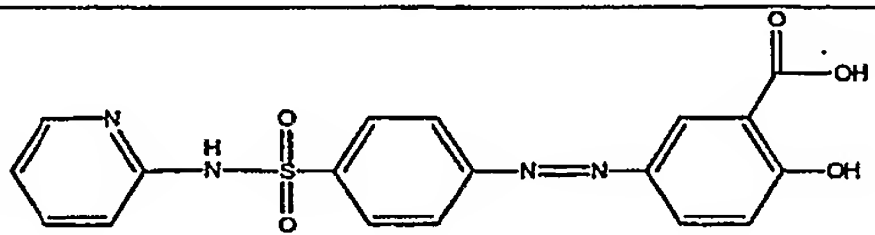
Surfaces coatings containing sets of 1, 2 or more ligands identified from the virtual screening based on the protein G:Fc and protein A:Fc models may be assembled and tested for their performance in biological assays. More specifically, the anchor site binding ligands are covalently attached to the poly(styrene-co-maleic anhydride) layer deposited

5   onto a standard Luminex microsphere used in the xMAP technology. These coated beads are used in a standard sandwich assay, such as the RAT IL2, and read on the standard Luminex 100 system.
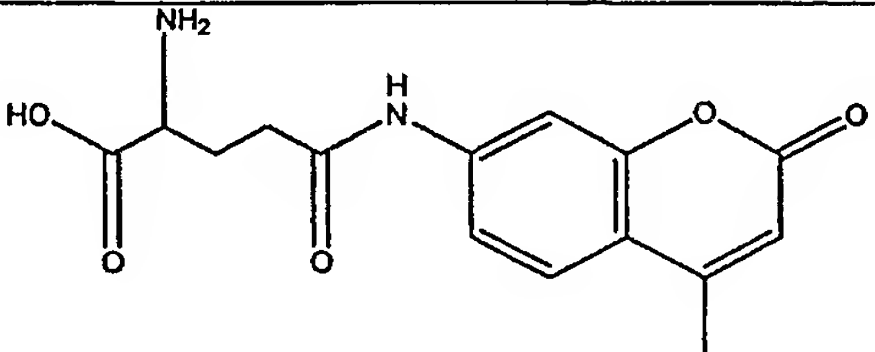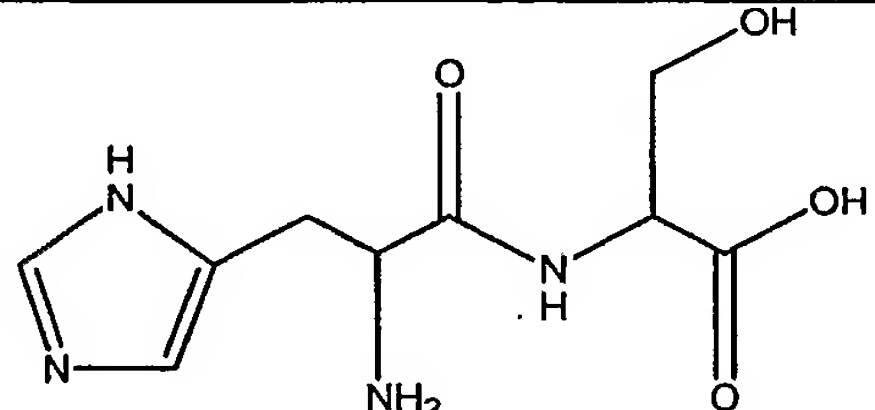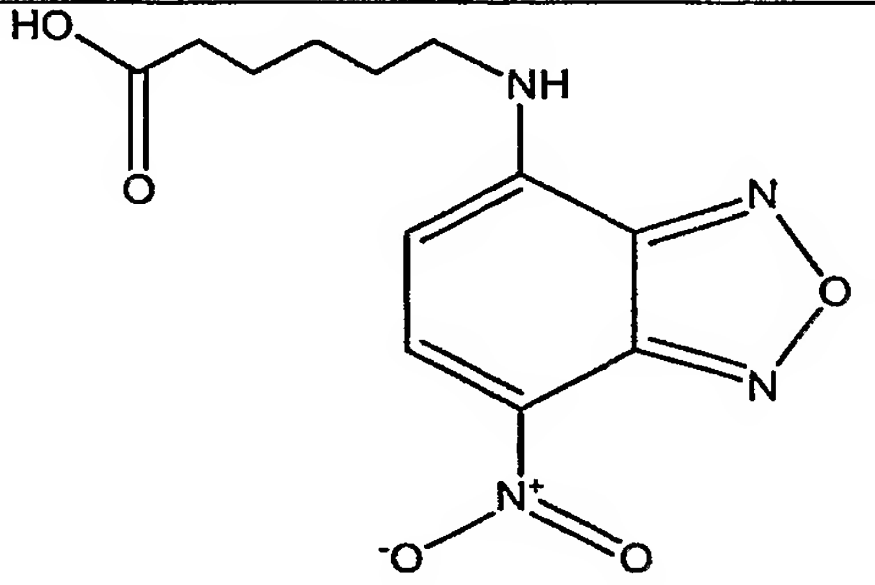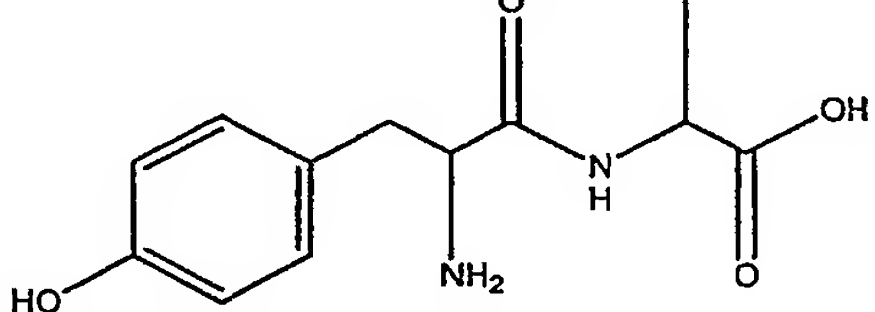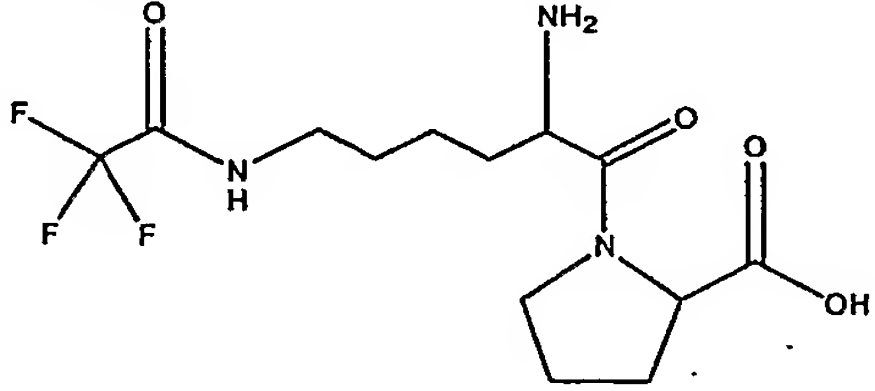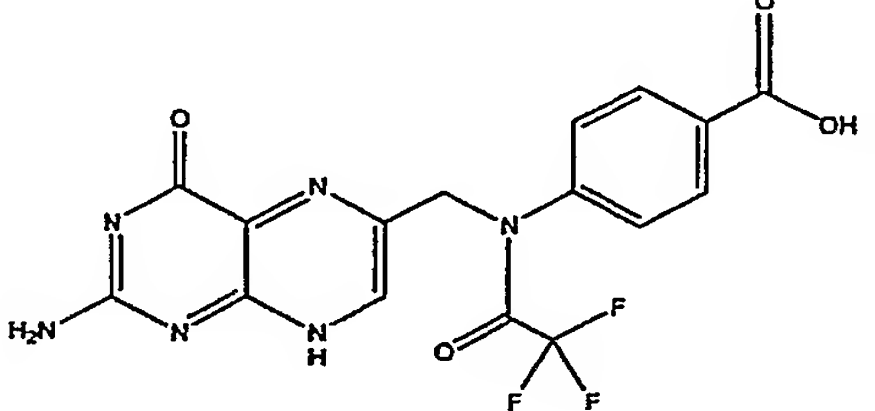
The proprietary, public domain and/or commercial softwares, the scoring function and

10   pharmacophore models that have been used in this example are continually updated and upgraded to refine the quality and the speed of the design.

Table 1

Name	Structure	Free binding energy (kJ/mol)
MYCOPHENOLIC ACID		-31.493
LAVENDUSTIN A		-50.929
PTEROIC ACID		-41.495
N10-(TRIFLUOROACETYL)PTEROIC ACID		-39.394
3-HYDROXY-4-(2-HYDROXY-4-SULFO-1-NAPHTHYL AZO)NAPHTHALENE-2-CARBOXYLIC ACID		-45.494

N-(4-NITROBENZOYL)-6-AMINOCAPROIC ACID		-40.411
5-(4-(2-PYRIDYLSULFAMOYL)PHENYLAZO)SALICYLIC ACID		-49.044
1,3,4,5-TETRAHYDROXYCYCLOHEXANECARBOXYLIC ACID 3-[3,4-DIHYDROXYCINNAMATE		-44.625
4'-(2-THIAZOLYLSULFAMOYL)-SUCCINANILIC ACID		-39.231
ASP-ALA BETA-NAPHTHYLAMIDE		-36.487
3-CARBOXYUMBELLIFERYL BETA-D-GALACTOPYRANOSIDE		-50.116
4-(N-[2,4-DIAMINO-6-PTERIDINYLMETHYL]-N-METHYLAMINO)BENZOIC ACID HEMIHYDROCHLORIDE		-35.845

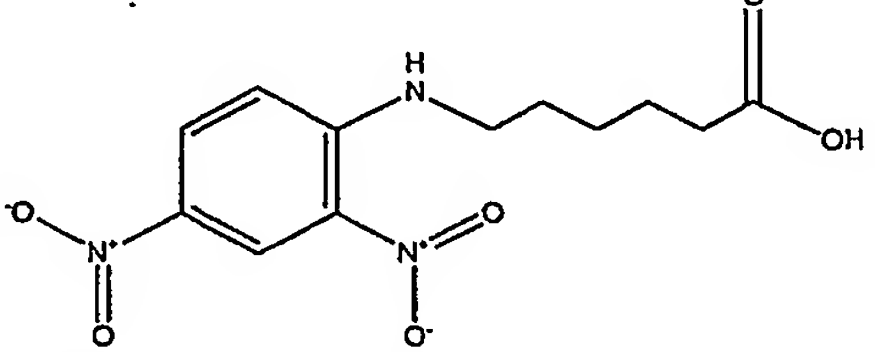|		
L-GLUTAMIC ACID GAMMA-(7-AMIDO-4-METHYLCOUMARIN)		-34.108
HIS-SER		-22.684
N-[7-NITROBENZ-2-OXA-1,3-DIAZOL-4-YL]AMINOHEXANOIC ACID		-31.769
TYR-ALA		-41.994
NEPSILON-TRIFLUOROACETYL-LYS-PRO		-30.499
N 10-(TRIFLUOROACETYL)PTEROIC ACID		-26.593
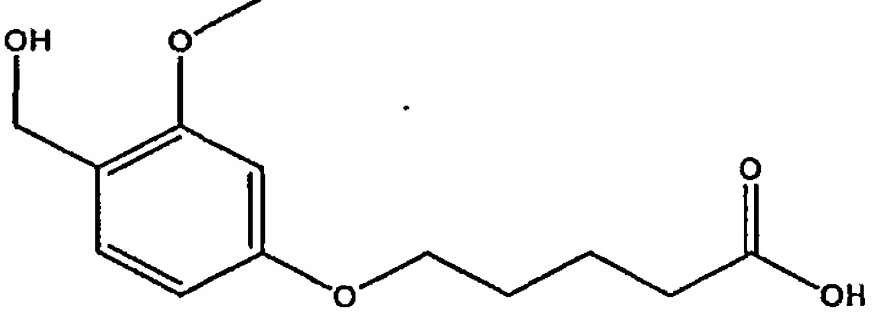
| 2,4-DINITROPHENYL-ALPHA-AMINOCAPROIC ACID | | -30.665 |
| 5-(4-HYDROXYMETHYL-3-METHOXYPHENOXY)VALERIC ACID | | -30.833 |

FIGURE 1

IgG2a
(MAB 231, 1IGT)

FIGURE 2

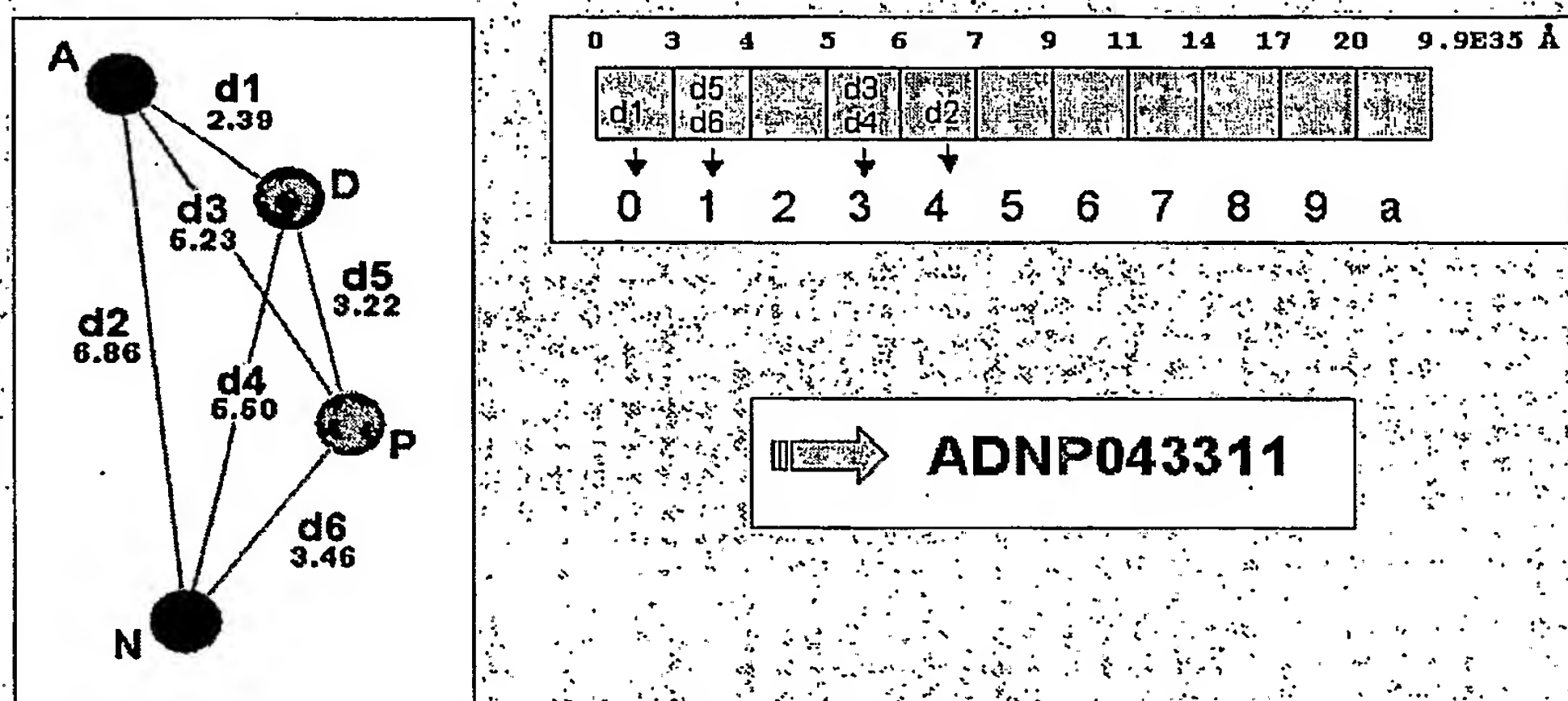**FIGURE 3**

5   **FIGURE 4A**   **FIGURE 4B**